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14. ABSTRACT

The goal of these studies is to develop and validate cell-penetrating bi-specific antibodies as an agent that can selectively inhibit the function of intracellular proteins. We have developed 3E10-AR441 bi-specific antibody to inhibit the function both ligand-dependent and independent forms of the androgen receptor (AR), key drivers of prostate carcinogenesis. Key accomplishments in this reporting period are the production of a prototype 3E10-AR441 bi-specific antibody, and demonstration of target engagement under denaturing and non-denaturing conditions, as well as in intact cell systems. The antibody inhibited the growth of prostate cancer cells. We will continue to characterize the biochemical lesions produced by this antibody, while developing higher affinity analogs with improved production characteristics.

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INTRODUCTION

This report covers research activities supported by DOD contract W81XWH-12-1-0534, entitled Cell-penetrating bispecific antibodies for targeting oncogenic transcription factors in advanced prostate cancer. The research is a collaborative effort between Michael Lilly, MD (Principal Investigator) and Richard Weisbart (Co-investigator). Dr. Lilly is based at the Hollings Cancer Center, Medical University of South Carolina, while Dr. Weisbart is at the Sepulveda VA, affiliated with the University of California Los Angeles. This contract was activated at MUSC on October 1, 2012. The reporting period is therefore from 10/1/2013 through 9/30/2014. Work under this contract began in Dr. Lilly's laboratory about November 1, 2012. Dr. Weisbart's group has worked from December, 2012, following the completion of a subcontract agreement between MUSC and Dr. Weisbart's institution.

Three specific aims were proposed. In the first, Dr. Weisbart's group would produce a prototype bispecific antibody targeting the androgen receptor (AR) and optimize its structure and production. The second specific aim, to be carried out by Dr. Lilly's laboratory, focuses on the biochemical and biologic properties of the bispecific antibody, through the use of biochemical and biologic assay systems. The final specific aim will examine the ability of the bispecific antibody to perturb the growth of prostate cancer cells in murine models. Additional studies will characterize PK/PD parameters of the antibody. Substantial progress has been made in the first two aims. Work has not yet started on the third aim.

BODY

1. Weisbart Group

During the past year we have focused on improving 3E10-AR441 bispecific single chain Fv. Although our initial construct penetrated prostate cancer cells *in vitro*, bound to the androgen receptor and was cytotoxic, two issues remained that needed improvement. 1. AR441 Fv has low affinity for the androgen receptor compared to the original monoclonal antibody. 2. The yield of 3E10-AR441 produced in *Pichia pastoris* is low.

A. Studies of alternate linkers to enhance binding affinities. The binding affinity of the Fv is largely determined by the linker between VL an VH which is responsible for bringing the two chains in close apposition to form an antigen binding site. The low affinity Fv was the result of using the standard (GGGGS)3 linker. Therefore, we constructed and produced AR441 Fv with different linkers that have been cited in the literature. We identified one linker that increased affinity of binding of AR441 Fv 4-fold by independent evaluation of binding affinity in Dr. Lilly's laboratory. We then produced a new construct of 3E10-AR441 with the higher affinity AR441. In preliminary studies, we have been able to produce the same amount of recombinant 3E10-AR441 as we obtained with the previous construct. This will now allow us to produce improved material for subsequent studies.

B. Production of bispecific antibodies. The second issue is the ability to produce sufficient recombinant protein for studies both *in vitro* and *in vivo*. Currently we are able to produce only 1 mg of 3E10-AR441 from 1 liter of *Pichia* supernatant. We recently compared two methods of producing 3E10-AR441 as a secreted protein in *Pichia pastoris* : a. the standard method of growing yeast in glycerol and then inducing production of protein with methanol, and b. simultaneous growth of *Pichia* and synthesis of protein in the presence of methanol without glycerol. The latter method is borrowed from industry where large yields of protein have been observed. This latter method was found to work well in our laboratory with other bispecific scFv antibodies containing 3E10 Fv, but was unsuccessful for 3E10-AR441. This result suggests that AR441 is toxic. In addition to toxicity, the conformation of the bispecific antibody influences its ability to be secreted. The conformation of the antibody complex is

determined to a great extent by the linker used to connect the two Fv fragments (3E10 Fv and AR441 Fv). We are just completing the process of producing different constructs of 3E10-AR441 with different linkers in an attempt to increase yield of protein, and we are ready to evaluate these constructs to optimize protein production.

Producing multiple molecular constructs and defining the optimum conditions for production and purification of recombinant protein is an arduous, labor intensive task that is usually the domain of industry. Nevertheless, we have accomplished our goal and will be able to produce small, but sufficient amounts of the higher affinity AR441 Fv for efficacy studies in Dr. Lilly's laboratory.

2. Lilly Group

A. Inhibition of AR genomic signaling by bispecific antibody. A key goal of specific aim #2 was to demonstrate that the 3E10-AR441 antibody can engage its target (AR) and interrupt AR-dependent signaling and effects. During the 01 year we developed a ARE-dependent luciferase reporter gene system in LNCaP cells, and used it to show that 3E10-AR441 can disrupt genomic AR signaling. During the 02 year we expanded these studies to show that the bispecific antibody can also disrupt AR signaling to an endogenous promoter. For these studies LNCaP cells were treated with DHT, with or without 3E10-AR441 antibody or control antibodies. Changes in the levels of PSA mRNA were measured by real time PCR (Fig. 1). The bispecific antibody completely prevented the ligand-dependent increase in PSA mRNA.

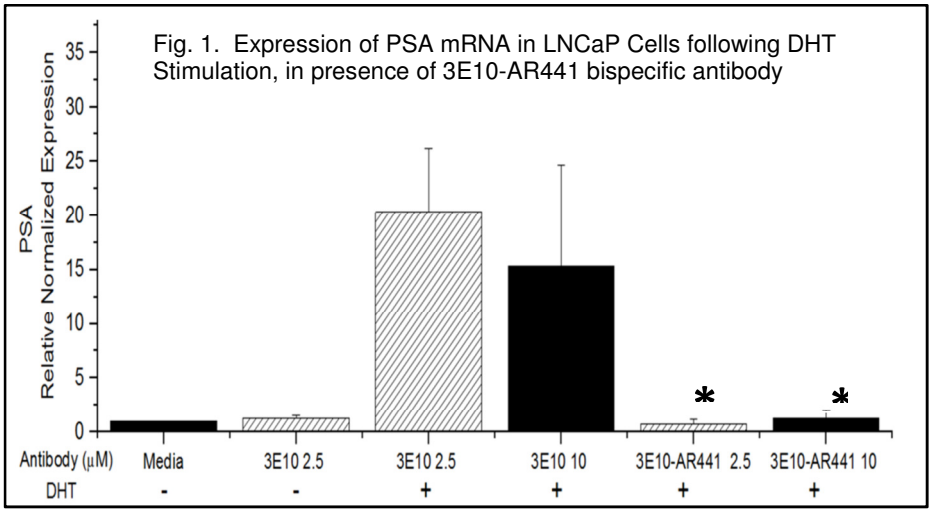

Fig. 1. Expression of PSA mRNA in LNCaP Cells following DHT Stimulation, in presence of 3E10-AR441 bispecific antibody

B. Inhibition of AR non-genomic signaling by bispecific antibody. AR can transduce signals in the cell membrane and cytoplasm immediately following ligand binding. These non-genomic signals do not require nuclear translocation and gene transcription. Non-genomic signaling can lead to release in calcium from intracellular pools within seconds of ligand application. 3E10-AR441 dramatically reduces DHT-dependent calcium release in LNCaP cells (Fig. 2). The 3E10 scFv alone did not significantly impair ligand-dependent calcium release.

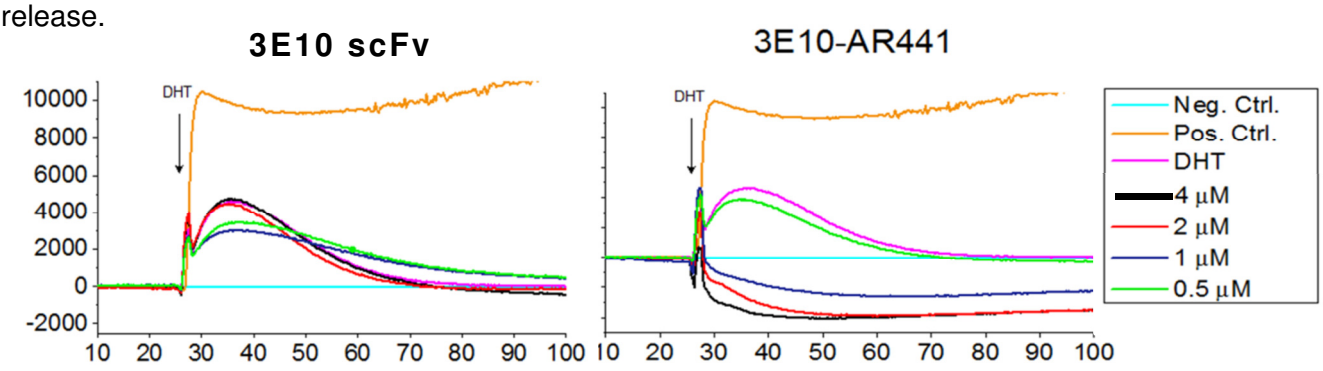

Fig. 2. Calcium release in LNCaP cells following DHT addition. Cells were pretreated for 1hr with various concentrations of 3E10 scFv, or the 3E10-AR441 bispecific antibody. X axis = time in seconds; Y axis = relative fluorescence

C. Binding affinity of parental MoAb AR441 and bispecific antibody 3E10-AR441. Initial attempts during the 01 year to measure the binding affinity of antibodies to AR via flow cytometery were unsuccessful due to non-specific binding of the bispecific antibody to the target cells under fixed, permeabilized conditions. During the 02 year we developed an ELISA-based method that has performed well. A commercially-available antibody to the N-terminus of human AR was used to coat wells of an ELISA plate. Following blocking, the capture antibody was charged with native AR derived from cell lysates. We used both endogenous WT AR (derived from a LNCaP cell lysate) and various mutant or splice variant ARs (derived from HEK cells transfected with AR expression plasmids). A HRP-tagged AR441 monoclonal antibody was then added, along with various competitor antibodies. The bound AR441-HRP antibody was then quantified with a colorimetric substrate. Calculations of Ki (approximately equal to Kd) were made by fitting a line to the competition curve.

The initial studies estimated the binding affinity of the parental monoclonal AR441 (Fig. 3). The studies produced values ranging from 0.3nM to 5nM (depending on which capture antibody was used). These values are very compatible with expected affinities for monoclonal antibodies. Interestingly, the affinity of the parental monoclonal differed among the various AR forms studied. The affinity of the MoAb AR441 for the Q640X mutant (premature stop codon, leading to loss of ligand binding domain [LBD]) was similar to that for the WT receptor (data not shown). However, the binding to several splice variants (similarly lacking LBD) was 50-70-fold less avid. This interesting observation suggests that there are conformational difference at the binding site between the WT receptor and the LBD-deficient splice variants. This conformational difference conceivable could permit the development of ligands

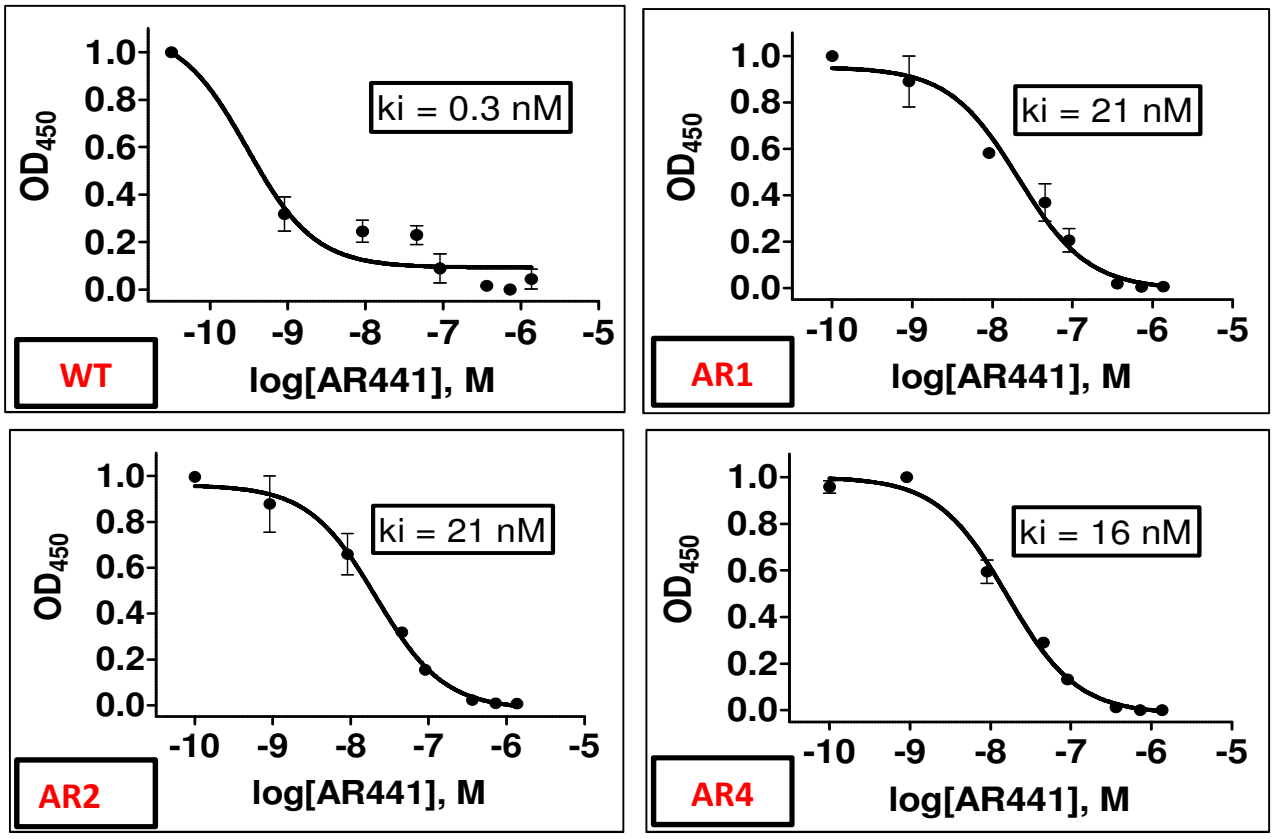

Fig. 3. Binding affinity of parent MoAb AR441 to WT AR and to AR splice variants lacking LBD.

selective for AR forms characteristic of CRPC.

Direct comparisons of the binding affinities obtained for the parental MoAb AR441 and for the 3E10-AR441 bispecific antibody revealed that the latter was about 25-fold less avid for the WT AR protein

(table 1). The discrepancy in binding affinities for the Q640X mutant was even greater. We have not yet measured binding affinities for the LBD-deficient splice variants. Interestingly, binding affinity of the isolated AR441 scFv for WT AR was more than 8-fold lower than that of the bispecific antibody. This is an encouraging result that suggests that there are favorable effects on binding resulting from the enhanced stabilization of the bispecific format.

The relatively low affinity of our first-generation AR441 scFv protein suggests the need for strategies to enhance antigen binding. Dr. Weisbart's group is approaching this problem through modification of linker sequences and module ordering within the expression plasmids (see above). Dr. Lilly's group will use antibody affinity maturation methods with a yeast surface display platform. The latter studies have just begun, using reagents obtained from the Pacific Northwest National Laboratory. Binding of antigen to positive-control yeast has been identified by multicolor flow cytometry. Based on discussions with several groups experienced with antibody affinity maturation, we expect to be able to improve the binding affinity of a second-generation bispecific antibody by 10-100-fold. In principle it should also be possible to select antibodies with relatively greater affinity for a mutant or splice variant over the WT receptor.

D. Development of a sandwich ELISA to detect 3E10-containing antibodies in biologic samples. In order to proceed to PK/PD studies in mice, we must have an assay capable of detecting very small amounts of 3E10-AR441 in biologic specimens. We envison a sandwich ELISA with an anti-3E10 capture antibody (rabbit polyclonal), and an anti-MycTAG-HRP or anti-MycTAG-biotin detection antibody. We have contracted with a commercial source for production of the anti-3E10 antibody and have received serum from the initial bleed. We purified antibodies by ammonium sulfate precipitation from the serum, followed by affinity purification on Protein A-agarose. We now have several milligrams of the purified preparation.

Our initial attempts to formulate an ELISA have met with technical challenges. The immune serum appears to have acquired an epitope that is recognized by the anti-MycTAG-HRP detection reagent. This is apparent by extremely high background signal in the ELISA, even in the absence of antigen. When the anti-3E10 purified immunoglobulin is studied by immunoblotting, using anti-MycTAG-HRP as the antibody to probe the blot, we see a strong signal with the anti-3E10 heavy chain derived from the immune serum, but not from the pre-immune serum. We anticipate several possible steps to eliminate this distressing artifact:

- Use the anti-3E10 for both the capture and detection antibody. This will require preparing a biotinylated antibody for the detection component. Since the anti-3E10 antibody consists of multiple clones reacting with different epitopes, we hope there would be enough variety to allow for multiple capture-detection pairs.
- Use an affinity column of anti-MycTAG antibody to absorb out the cross-reacting protein from the anti-3E10 antibody

Addition of mouse serum to the blocking buffer has not helped increase specific signal. This suggests that the problem is not promiscuous binding of mouse antibody by the capture antibody, but rather binding of a subset of the capture antibody immunoglobulins by the anti-MycTAG mouse monoclonal.

We are currently (October, 2014) exploring the second alternative. As assay development proceeds we are also pursuing an IACUC approval for a pilot PK/PD experiment.

E. Development of cell lines stably expressing WT and mutant ARs. To most precisely catalogue the AR forms that react with 3E10-AR441 we will still need to express these individually in mammalian cells that lack substantial amounts of endogenous AR. Since BPH1 cells were not satisfactory we

have created transient and stable transfectants of HEK cells for this purpose. HEK/AR WT and HEK/AR Q640X cell lines have already been prepared. Other cell lines containing AR splice variants are under development. We will transduce these cell lines to express the ARE-driven luciferase gene, allowing us to have only 1 form of AR in each cell line, for both immunologic study and functional analysis. We are also making these into LNCaP cells as a backup plan. LNCaP already have a WT and T877 mutant AR alleles, but are relatively easy to transduce. They may allow us to obtain some data if the HEK cell lines cannot be used to reconstitute a DHT→AR→luciferase signaling axis.

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 02

1. Weisbart Group
   A. Design, synthesis, and production of the 3E10-AR441 bispecific antibody variants with multiple new linker sequences
   B. Production of control scFv and bispecific antibodies scFv3E10, 3E10-3G5, and scFvAR441 for use by Lilly laboratory

2. Lilly Group
   A. Demonstration that 3E10-AR441 inhibits androgen-dependent genomic and genomic AR-dependent signaling. Genomic signaling studies used LNCaP cells engineered to expess firefly luciferase under the control of a synthetic ARE. Similar studies were also performed with intact LNCaP cells stimulated with DHT, to show inhibition of PSA mRNA expression as an example of genomic signaling through an endogenous receptor. Controls were 3E10, 3E10-3G5, enzalutamide.
   B. Demonstration that 3E10-AR441 can inhibit non-genomic, AR-dependent signaling. These studies used LNCaP cells stimulated with DHT, with a readout of calcium release detected fluorometrically over time. Controls included ionomycin (positive control), enzalutamide, 3E10
   C. Demonstration that 3E10-AR441 bispecific antibody can bind to wild-type and LBD-deficient splice variants of the AR under “native” conditions, using an immunoprecipitation assay with a protein L-coated solid phase
   D. Development of a competitive EIA assay to measure binding affinity of antibodies to the AR441 epitope. We have used the assay to measure of binding affinities of parental MoAb AR441 to WT, truncated, and splice variant ARs. Binding affinities of 3E10-AR441 bispecific antibody to WT and truncated ARs have also been accomplished.
   E. Development and characterization of test cell lines for study of 3E10-AR441:
      i) LNCaP-ARELuciferase
      ii) LNCaP/WT AR
      iii) LNCaP/AR Q640X
      iv) HEK/WT AR
      v) HEK/AR Q640X
      vi) HEK/AR1
      vii) HEK/AR3
      viii) HEK/AR4

REPORTABLE OUTCOMES

1. The 3E10-AR441 bispecific antibody will enter prostate cancer cells and localize predominately in the nucleus
2. The 3E10-AR441 bispecific antibody can bind to a variety of AR forms, including receptors lacking the ligand-binding domain, under non-denaturing conditions.

3. The 3E10-AR441 bispecific antibody inhibits genomic and non-genomic signaling by the wild type (and presumably T877 mutant) AR forms in LNCaP cells.
4. The AR-V1, AR-V7, and AR-2b splice variants have a different conformation in the vicinity of the AR441 epitobe than do the WT and Q640X mutant receptors
5. The 3E10-AR441 bispecific antibody has a lower binding affinity for both WT and truncated ARs than does the parental MoAb AR441.
6. Dr. Goicochea-Papaffava is preparing a manuscript describing these findings, for submission to a chemistry-oriented journal.

DELIVERABLES

1. Rabbit polyclonal antibody to scFv 3E10, purified by ammonium sulfate precipitation and Protein A affinity chromatography, approximately 8mg
2. Poster describing studies up to May, 2014, presented at the Prostate Cancer Research Symposium, Hollings Cancer Center, Medical University of South Carolina, 5/15/2014
3. Multiple cell lines containing WT or variant AR constructs

CONCLUSIONS

1. The bispecific 3E10-AR441 antibody has performed as expected in tissue culture experiments, by entering cells, translocating to the nucleus, and blocking androgen-dependent genomic and non-genomic signaling through the AR. The antibody binds to both wild-type and LBD-mutant ARs, demonstrating robust target engagement.
2. The binding affinity of the 3E10-AR441 is probably several-fold lower than that of the parental monoclonal AR441. This may require much larger amounts of antibody than expected for a significant *in vivo* biologic effect.
3. Further protein engineering of the 3E10-AR441 antibody is probably needed to 1) enhance the binding affinity to the target protein, and 2) to improve yields from the current yeast expression system
4. The studies accomplished to date by both the Lilly and Weisbart laboratories represent a substantial completion of the propose studies for months 1-24 under specific aims #1, 2, as outlined in the approved Statement of Work. Studies under specific aim #3 as outlined in the modified SOW are beginning, as indicated by our development of an ELISA to measure 3E10-AR441 in biologic samples.

REFERENCES

none

APPENDICES

none